IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No. :

U.S. National Serial No. :

Filed :

PCT International Application No. : PCT/FR02/01943

VERIFICATION OF A TRANSLATION

I, the below named translator, hereby declare that:

My name and post office address are as stated below;

That I am knowledgeable in the French language in which the below identified international application was filed, and that, to the best of my knowledge and belief, the English translation of the international application No. PCT/FR02/01943 is a true and complete translation of the above identified international application as filed.

I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application issued thereon.

Date: November 27, 2003

B. Parish

Full name of the translator : Elaine Patricia PARRISH

For and on behalf of RWS Group plc

Post Office Address : Europa House, Marsham Way,

Gerrards Cross, Buckinghamshire,

England.

Lipoxygenase overexpression in plants and reduction in plant sensitivity to diseases and to attacks from pathogenic organisms

The present invention relates to methods for reducing plant sensitivity to diseases and attacks from pathogenic organisms. The methods according to the invention consist in overexpressing a lipoxygenase in plants so as to reduce their sensitivity to diseases and attacks. The invention also relates to expression cassettes, to vectors and to transformed plants used in the methods according to the invention.

Lipoxygenases (LOXs) are ubiquitous enzymes in higher plants and mammals. They catalyze the dioxygenation of polyunsaturated fatty acids containing a (*Z,Z*)-1,4-pentadiene unit. In plants, substrates for the enzyme are linoleic acid (C18:2) and linolenic acid (C18:3), which are two major constituents of cell membranes. These polyunsaturated fatty acids are generally complexed in the form of membrane-bound phosphoglycerolipids and are only accessible to LOXs after the action of a phospholipase type A_2 or lipolytic acyl hydrolases. Recent studies suggest, however, that certain LOXs may, under certain circumstances, oxidize esterified fatty acids and membrane-bound lipids (1-4). Arachidonic acid, which is not detectable in plants, but which is part of the membrane-bound constituents in oomycetes, is also a substrate for plant LOXs (5).

LOXs are classified according to the position of the carbon onto which the molecular oxygen is preferentially inserted. In plants, 13-LOXs and 9-LOXs are distinguished; the same enzyme can, however, use one or other position, indifferently or else with a preference for a position. This specificity can be modified according to the conditions of pH and of O_2 concentration of the medium (4). The position specificity of a LOX cannot be directly predicted according to its primary sequence, even though some structural elements related to this property are now known (6, 7).

The products formed in the reaction are fatty acid hydroperoxides, which are very reactive and capable of causing, via free-radical reactions, degradation of the major constituents of the cell (lipids, proteins, nucleic acids) (8). Fatty acid hydroperoxides are rapidly converted into a series of compounds having diverse biological activities. All the products derived from polyunsaturated fatty acids via oxygenation are collectively referred to as oxylipins.

Plant LOXs have been associated with varied physiological processes, on the basis of profiles of gene expression and of enzyme activity. Thus, it has been proposed that LOXs are involved in regulating seed maturation, germination, fruit maturation, and leaf and

flower senescence. The precise contribution of LOXs to these processes remains to be determined however. An important role is also attributed to LOXs in response to stress, in particular injury, and parasitic attacks (4, 5, 9). Strong induction of LOX gene expression is thus measured in many monocotyledon or dicotyledon plants when they interact with bacteria, viruses or fungi, and also after mechanical injury or injury caused by insects on the leaves.

This diversity in the biological functions may be provided due to the presence of various isoenzymes, exhibiting very varied mechanisms of regulation and tissue and subcellular locations, according to the species and isoforms under consideration (5). Added to this is the diversity of the oxylipins generated from the products of the LOX, which is modulated according to the type of hydroperoxide formed and to the nature of the enzymes which metabolize them.

The fatty acid hydroperoxides generated by the LOX are in fact converted according to several distinct enzyme pathways (4). The hydroperoxide lyase (HPL) pathway catalyzes cleavage of 13- or 9-hydroperoxides of fatty acids so as to achieve the synthesis of volatile C6- or C9-aldehydes and of C12 or C9 short-chain acids, such as 12-oxo-*trans*-9-dodecenoic acid, the precursor of traumatic acid. C6-aldehydes play an important role in plant fragrance, but some, such as *trans*-2-hexenal, also have antimicrobial properties (10). It has recently been shown that *trans*-2-hexenal can act as a signal molecule for defense gene activation (11). Traumatic acid, also referred to as wound hormone, is thought to have a role in tissue healing by promoting cell division at sites of injury (5).

A second enzyme pathway involved in the metabolism of fatty acid hydroperoxides produced by LOX concerns allene oxide synthase (AOS). This enzyme catalyzes the dehydration of 13-hydroperoxilinolenic acid and forms an allene oxide, which is the precursor of the jasmonic acid. Jasmonic acid is a key molecule of plant signaling mechanisms for activating many defense genes (12), among which are the genes encoding protease inhibitors active against insects, and also many PR proteins (chitinase, defensin, thionin, glucanase). The role of jasmonates as a signal molecule has recently been underlined by various experiments showing that *Arabidopsis* mutants or tomato mutants affected in terms of the biosynthesis of these molecules or the distinction thereof, exhibit a deficiency in defense gene induction (13), greater sensitivity to organisms which are normally nonpathogenic for wild-type plants (14) or reduced resistance to insects (15). A third enzyme pathway for conversion of hydroperoxides concerns the formation of divinyl ether fatty acids. These compounds, which can be formed from 13- or 9-hydroperoxides,

have been isolated from various plants. The first divinyl ether synthase (DES) was recently cloned in the tomato (16). The divinyl ether fatty acids produced from 9-hydroperoxides,colneleic acid and colnelenic acid, exhibit antifungal properties, in particular with respect to *Phytophthora infestans* (17).

Other modifications of the products of the reaction catalyzed by LOX concern epoxidations with epoxygenases and peroxygenases. This metabolic pathway allows the synthesis of molecules with antimicrobial activity and may be involved in the synthesis of cutin monomers, which, besides their structural function, can also induce the activity of defense genes (18).

The fatty acid hydroperoxides produced by LOX can, finally, generate the formation of free radicals involved in membrane degradation mechanisms associated with cell death (5).

All these data therefore show that the initial activity of LOX is essential for the synthesis of a set of molecules, some of which exhibit antimicrobial activities or are involved in the signaling leading to defense.

LOX activity increases notably in tobacco in response to elicitors (19, 20). The isolation of a LOX complementary DNA clone, in particular pTL-J2 (21), has made it possible to characterize the corresponding tobacco gene, called *LOX1*. DNA/RNA hybridization experiments (Northern hybridization) have shown that *LOX1* is expressed in tobacco cells in culture subsequent to the application of elicitor and in tobacco plants inoculated with the oomycete *Phytophthora parasitica nicotianae, Ppn* (22). The transcripts corresponding to this gene are not detectable in normal plants or untreated cells. A biochemical study shows that, *in vitro*, the LOX of elicited tobacco cells allows the production of 9- and 13-hydroperoxides of fatty acid, with preferential insertion of the molecular oxygen in the 9-position (20).

Subsequent to root inoculation of tobacco with *Ppn*, strong induction of expression of the gene is measured. In the case of an incompatible interaction (resistant plant/avirulent race of microorganism), early induction of the activity of the gene is measured, with a maximum accumulation of transcripts at 24 hours (22). Induction of the gene is later and smaller in amplitude in the case of a compatible interaction (sensitive plant/virulent race), as has also been observed in other gene-for-gene models, such as interactions between the tomato and *Pseudomonas syringae* (23), rice and *Magnaporthe grisea* (24), and the potato and *Phytophthora infestans* (25). Expression of the *LOX1* gene is not detected in the tissues of normal tobacco plants, with the exception of flowering plants for which the LOX

transcripts are detected in low amount in the petals and sepals, and young germinations (22, 26). In the latter case, transient expression of the *LOX1* gene is detected between the second and fourth days after the beginning of germination.

Villalba et al. (27) have shown that an elicitor glycoprotein isolated from *Ppn* walls, called CBEL for Cellulose Binding Elicitor Lectin, induces the *LOX1* gene when it is infiltrated into the foliar mesophyll of tobacco plants. The *LOX1* transcripts appear during the first four hours following infiltration and reach a maximum level at 12 hours. A polysaccharide derived from marine algae, λ-carraghenan, also induces the *LOX1* gene when it is applied to tobacco plants by infiltration into the foliar mesophyll (28).

In tobacco cells, expression of the *LOX1* gene is detected subsequent to eliciting treatments. In the case of *Ppn* elicitor (wall extract), induction of expression of the *LOX1* gene is detected within the first two hours after treatment, with a maximum accumulation at 24 hours (22). Cryptogein, an elicitor peptide of *Phytophthora cryptogea*, an oomycete for which tobacco is not a host, and *Colletotrichum lindemuthianum* endopolygalacturonase, also make it possible to induce the *LOX1* gene (26). It has been shown, moreover, that induction of the *LOX1* gene occurs earlier than that of defense genes such as those encoding PR proteins, namely chitinases and ß1-3-glucanases, suggesting that the LOX pathway has a potential role in the signal transduction triggering the defense reactions (26). *LOX1* expression in cells is also inducible with methyl jasmonate (22, 29), thus indicating possible self-amplification of this pathway, whereas no accumulation of LOX transcripts is detected after the application of salicylic acid (22).

Analysis of expression of the *LOX1* gene shows that the induction of LOX constitutes an early response of the plant to infection with *Ppn*, suggesting a role in the establishment of resistance. This potential role has been confirmed through obtaining transgenic plants expressing the complementary DNA of the gene in an antisense orientation. In fact, these plants, originating from the 46-8 line normally resistant to *Ppn* race 0, exhibit greatly reduced levels of LOX activity and have lost their ability to trigger the incompatible reaction (30, 31). This experiment clearly shows that expression of the LOX gene is necessary to establish the resistant state in the gene-for-gene interaction between tobacco and *Ppn*. The LOX antisense plants are also more sensitive to another pathogenic fungus of tobacco, *Rhizoctonia solani* (30, 31). In parallel, Rustérucci et al. (32) have shown that 9-hydroperoxides accumulate during the hypersensitive-type response of tobacco to cryptogein, and that this accumulation is necessary for the development of this reaction. In another Solanacea, the potato, oxylipins derived from the 9-LOX pathway, in

particular colneleic acid and colnelenic acid, preferentially accumulate in cells in culture in response to a *P. infestans* elicitor (33). All these data suggest a major role for the 9-LOX pathway in Solanaceae, including tobacco, in the response to pathogenic agents, in particular oomycetes. In these plants, the 13-LOX pathway contributes to the response to pathogenic agents, as shown by the early synthesis of jasmonates (29); it is also involved in the response to injury and to insects. Thus, potato plants expressing a 13-LOX antisense construct have been found to be more sensitive to insect attack (34).

Few attempts at overexpressing a LOX in plants have been reported in the literature. In fact, the expression of a LOX poses questions of feasibility due to the potentially toxic properties of LOXs, if they directly oxidize biomembranes, and of the products which they form.

In tobacco, introduction of soybean LOX2 under the control of a chimeric promoter, formed by fusion of the cauliflower mosaic virus (CaMV) 35S promoter and an enhancer isolated from the alfalfa mosaic virus, has made it possible to produce transgenic calluses exhibiting a LOX activity which is approximately 2 times greater than that of calluses transformed with the vector lacking LOX sequence. These calluses are capable of forming 3 to 6 times as many volatile aldehydes, products of the 13-LOX pathway via a 13-HPL, as the control calluses. Transgenic plants were regenerated from these calluses but, although these plants exhibited a high accumulation of the heterologous protein, their LOX activity is no different from that of control plants. However, they form larger amounts of volatile aldehydes than control plants (35). More recently, a 13-LOX specific for the lipid bodies of cucumber seeds has been expressed in tobacco (36). The heterologous protein accumulates throughout the plant, in transgenic tobacco plants, and in particular in the seeds, where it is essentially located in the lipid bodies as in the cucumber. Its presence results in a qualitative modification of the LOX activity of the transgenic plants, both *in vitro* and *in vivo*. An *Arabidopsis* chloroplast 13-LOX, AtLOX2, has been used in a construct in the sense orientation, under the control of the CaMV 35S promoter, to transform garden arabis plants. The authors focus, however, on the transformation events which led to a decrease in LOX activity by cosuppression (37). Finally, in the lentil, transient transformation of protoplasts with a construct containing a lentil LOX under the control of the CaMV 35S promoter produced a 20% increase in the LOX activity of these protoplasts (38). None of the transformed plants or cultures described above was tested with respect to a response relating to attack from pathogenic agents. Moreover, the experiments carried out essentially concern 13-LOXs.

In addition, the hypotheses concerning the participation of lipoxygenases in plant defense mechanisms are essentially based on the biological activities of certain oxylipins (39). Now, the biosynthesis of most oxylipins, and in particular jasmonic acid and the volatile aldehydes derived from the 13-LOX pathway, and the divinyl ethers derived from the 9-LOX pathway, requires the activity of enzymes which metabolize the LOX products, such as AOS, HPL or DES, beyond the LOX itself. Expression of the genes corresponding to these enzymes is often inducible. In the tomato and *Arabidopsis thaliana*, the expression genes encoding AOS and HPL is wound-inducible (40-42). Consequently, the action of only lipoxygenases does not therefore appear to be sufficient to trigger plant resistance mechanisms.

Cultivated plants are subjected to attack by many pathogenic organisms, such as viruses, bacteria and fungi, but also pests such as insects. These attacks weaken the plants and decrease their crop yields. There is therefore a considerable need to increase the resistance mechanisms of plants and to decrease their sensitivity to diseases and attacks from parasitic or pathogenic organisms. The mechanisms of responsive plants to attacks from pathogenic agents have been the subject of many studies. It is now commonly accepted that the lipoxygenase pathway in plants contributes to their system of defense and to establishing a state of resistance through the oxylipin pathway in particular.

However, knowledge of these metabolic pathways and of the lipoxygenases has not made it possible to develop methods for directly increasing plant resistance.

This problem is solved by the present invention since it has now been noted that overexpression of a lipoxygenase in plants directly reduces plant sensitivity to diseases and attacks from pathogenic agents. Unexpectedly, and although lipoxygenase is part of complex metabolic pathways, overexpression of a lipoxygenase in plants is sufficient to improve plant response to attacks from pathogens. In addition, overexpression of the lipoxygenase does not substantially affect the phenotype of the transformed plants, besides the novel acquired properties.

The present invention therefore consists in overexpressing a lipoxygenase in plants so as to reduce plant sensitivity to diseases and attacks from pathogenic organisms or pests. A subject of the invention is also preferred expression cassettes for overexpressing the lipoxygenase in plants, and also plant cells and transformed plants.

Description of the sequence listing

SEQ ID No. 1: Tobacco lipoxygenase (LOX1).

SEQ ID No. 2: CaMV 35S promoter – coding sequence of tobacco *LOX1* gene – nos terminator expression cassette.

SEQ ID Nos. 3-6: Primers for PCR.

Description of the invention

The present invention relates to a method for reducing plant sensitivity to diseases and attacks from pathogenic organisms. This method consists in overexpressing a lipoxygenase in these plants.

The term "lipoxygenase" means an enzyme which catalyzes the dioxygenation of polyunsaturated fatty acids containing a (Z,Z)-1,4-pentadiene unit. In plants, the substrates for the enzyme are linoleic acid (C18:2) and linolenic acid (C18:3), which are two main constituents of cell membranes.

The term "overexpression" means that the lipoxygenase is expressed at a level greater than the expression level observed in a reference plant (not induced). This overexpression results in greater accumulation of the lipoxygenase gene transcripts and of the lipoxygenase itself, and in an increased lipoxygenase specific activity in the tissues of the plant. To reduce plant sensitivity to diseases and attacks from pathogenic organisms, it is important for the level of expression of the lipoxygenase to be greater than that of a reference plant when the attack on the pathogenic organism occurs.

Overexpression of the lipoxygenase makes it possible to reduce plant sensitivity to disease and attacks from pathogenic organisms. The expression "attack from pathogenic organisms" is in particular attacks on the plants from viruses, bacteria, fungi, oomycetes or insects.

In a preferred embodiment, the method consists in constitutively overexpressing a lipoxygenase in plants. The term "constitutively" denotes temporal and spatial expression of the lipoxygenase in plants in the methods according to the invention. The term "constitutively" means the expression of a lipoxygenase in the tissues of the plant throughout the life of the plant and in particular during its entire vegetative cycle. In a first embodiment, the lipoxygenase is expressed constitutively in all plant tissues. In a second embodiment, the lipoxygenase is expressed constitutively in the roots, the leaves, the stems,

the flowers and/or the fruits. In another embodiment of the invention, the lipoxygenase is expressed constitutively in the roots, the leaves and/or the stems.

In a particular embodiment of the invention, the lipoxygenase is a lipoxygenase which is "inducible" in a reference plant. In the present invention, lipoxygenase which is "inducible" means a lipoxygenase which is not expressed, or is expressed at very low levels, and the expression of which is greatly induced in response to elicitors in the response to stress, to wounds, and in particular to diseases and attacks from pathogenic organisms.

In a preferred embodiment of the method according to the invention, the lipoxygenase preferably has 9-lipoxygenase activity. Lipoxygenases (LOX) are classified according to the position of the carbon onto which the molecular oxygen is preferentially inserted. In plants, 13-LOXs and 9-LOXs are distinguished. Methods for determining the specificity of lipoxygenase activity are described in the literature (Fournier et al., *Plant J.* 3:63-70, 1993; Hornung et al., PNAS 96:4192-4197, 1999; Rustérucci et al., *J. Biol. Biochem.* 274:36446-36455, 1999).

Any lipoxygenase the overexpression of which in plants makes it possible to reduce plant sensitivity to diseases and attacks by pathogen organisms can be used in the methods according to the invention. Lipoxygenases are known to those skilled in the art, and other lipoxygenases can be identified using known techniques. By way of example, mention may in particular be made of potato (Kolomoiets M.V. et al., *Plant Physiol.* 124:1121-1130, 2000), tomato (Genbank AY008278) and potato tuber (Royo et al., *J.Biol.Chem.*, 271:21012-21019, 1996; Casey, R., *Plant Physiol.*, 107:265-266, 1995), lipoxygenases, almond seed lipoxygenase (Mita et al., *Eur. J. Biochem.*, 268:1500-1507, 2001) and barley seed lipoxygenase (Van Mechelem et al., Biochem. Biophys. Acta, 1254:221-225, 1995).

The lipoxygenase is preferably a plant lipoxygenase.

In a particular embodiment of the invention, it is a Solanacea plant lipoxygenase. Among the Solanacea plants, mention may in particular be made of tobacco, tomato, potato or else pepper.

In another preferred embodiment of the invention, the lipoxygenase exhibits at least 80% homology with the tobacco lipoxygenase 1 (LOX1) of SEQ ID No. 1. Advantageously, the percentage homology will be at least 80%, 85%, 90%, 95%, and preferably at least 98%, and more preferably at least 99%, with respect to SEQ ID No. 1. The term "homolog" denotes a polypeptide which may exhibit a deletion, an addition or a substitution of at least one amino acid. The methods for measuring and identifying homologies between polypeptides or proteins are known to those skilled in the art. Use may, for example, be

made of the UWGCG package and the BESTFITT program for calculating homologies (Devereux et al., *Nucleic Acid Res.* 12, 387-395, 1984). Preferably, the homologous lipoxygenases conserve the same biological activity as the tobacco lipoxygenase (LOX1) of SEQ ID No. 1. Preferably, these polypeptides therefore have a lipoxygenase activity, and even more preferably 9-lipoxygenase activity.

In an even more preferred embodiment, the methods according to the present invention use the lipoxygenase of SEQ ID No. 1.

Overexpression of the lipoxygenase in plants is carried out by transforming the plants with, or by applying to the plants, a molecule for stimulating lipoxygenase synthesis in the plant.

In a preferred embodiment of the invention, the lipoxygenase is overexpressed by integration into the genome of the plants of an expression cassette comprising a sequence encoding a lipoxygenase under the control of a promoter which is functional in plants.

According to the invention, the term "promoter" means the noncoding region of a gene involved in binding the RNA polymerase and with other factors which are responsible for initiating and regulating transcription leading to the production of an RNA transcript. The plant promoters which can be used in the methods according to the present invention are widely described in the literature.

The promoter is preferably a promoter which is constitutive in plants. The constitutive promoters which can be used in the method according to the invention are also well known to those skilled in the art.

As a promoter regulatory sequence in plants, use may be made of any promoter sequence for a gene which is naturally expressed in plants, such as, for example, "constitutive" promoters of bacterial, viral or plant origin. Mention may be made of bacterial promoters such as that of the octopine synthase gene or of the nopaline synthase gene, viral promoters, such as the cauliflower mosaic virus 35S promoter or the CSVMV promoter (WO 97/48819), and promoters of plant origin such as the histone gene promoter (EP0507698) or the promoter of a rice actin gene (US 5,641,876). According to the invention, use may in particular be made, in combination with the promoter regulatory sequence, of other regulatory sequences, which are located between the promoter and the coding sequence, such as transcription activators (enhancers).

In a preferred embodiment of the invention, the constitutive promoter is the cauliflower mosaic virus 35S promoter.

In another embodiment of the invention, the constitutive expression or the overexpression of the lipoxygenase is obtained by transforming the plants so as to place a constitutive promoter or an enhancer sequence upstream of or in the vicinity of the lipoxygenase gene in the plants. Use may in particular be made of any regulative sequence for increasing the level of expression of lipoxygenase in plants.

Preferably, the lipoxygenase is overexpressed in the stems, the leaves and/or the roots of the plant.

According to the invention, the term "plant" means any differentiated multicellular organism capable of photosynthesis, in particular monocotyledons or dicotyledons, more particularly crop plants which may or may not be intended for animal or human food, such as maize, wheat, barley, sorghum, rapeseed, soybean, rice, cane sugar, beetroot, tobacco, cotton, etc.

Overexpression of the lipoxygenase can be obtained in any plant according to methods known to those skilled in the art.

In a particular embodiment of the invention, the plants are chosen from Solanaceae plants. Among the Solanacea plants mention will in particular be made of tobacco, tomato, potato or else pepper.

In a preferred embodiment of the invention, the lipoxygenase is overexpressed by integration into the plant genome of an expression cassette comprising a sequence encoding a lipoxygenase under the control of a promoter which is functional in plants. A polynucleotide encoding a lipoxygenase is inserted into an expression cassette using cloning techniques well known to those skilled in the art. This expression cassette comprises the elements required for transcription and translation of the sequences encoding the lipoxygenase in plants. Advantageously, this expression cassette comprises both elements for making the transformed plants produce the lipoxygenase and elements required for regulating this expression. The present invention also relates to preferred expression cassettes which can be used in the methods according to the invention.

In one embodiment, the present invention relates to expression cassettes which are functional in plant cells and plants, comprising a promoter having constitutive activity in plants, controlling the expression of a polynucleotide encoding a lipoxygenase at least 90% homologous to the lipoxygenase of SEQ ID No. 1. Advantageously, the percentage homology will be at least 80%, 85%, 90%, 95%, and preferably at least 98%, and more preferably at least 99%, with respect to SEQ ID No. 1. Preferably, this polynucleotide encodes a lipoxygenase having 9-lipoxygenase activity. More preferably, this

polynucleotide encodes the lipoxygenase of SEQ ID No. 1. In a particular embodiment of the invention, the promoter is the cauliflower mosaic virus 35S promoter.

The expression cassettes according to the invention preferably comprise a terminator sequence. These sequences allow termination of transcription and polyadenylation of the mRNA. Any terminator sequences which is functional in plants can be used. For expression in plants, use may in particular be made of the *Agrobacterium tumefaciens nos* terminator, or else terminator sequences of plant origin, such as, for example, the histone terminator (EP 0 633 317), the CaMV 35S terminator or the *tml* terminator. The terminator sequences can be used in monocotyledon and dicotyledon plants.

The techniques for construction of these expression cassettes are widely described in the literature (see in particular Sambrook et al., Molecular Cloning : A Laboratory Manual, 1989).

Advantageously, the expression cassettes according to the present invention are inserted into a vector for replicating them or for transforming plants.

The present invention also relates to vectors for transforming plants, comprising at least one expression cassette according to the present invention. This vector may in particular consist of a plasmid or of a virus into which is inserted an expression cassette according to the invention. Many vectors have been developed for transforming plants with *Agrobacterium tumefaciens*. Other vectors are used for transformation techniques not based on the use of *Agrobacterium*. These vectors are well known to those skilled in the art and widely described in the literature. Preferably, the vectors of the invention also comprise at least one selection marker. Among the selection markers, mention may be made of genes for resistance to antibiotics, such as the *nptII* gene for kanamycin resistance (Bevan et al., Nature 304:184-187, 1983) and the *hph* gene for hygromycin resistance (Gritz et al., Gene 25:179-188, 1983). Mention will also be made of genes for tolerance to herbicides, such as the *bar* gene (White et al., NAR 18:1062, 1990) for tolerance to bialaphos, the EPSPS gene (US 5,188,642) for tolerance to glyphosate or else the HPPD gene (WO 96/38567) for tolerance to isoxazoles.

The present invention also relates to vectors comprising an expression cassette according to the invention.

A subject of the invention is also a method for transforming plants with an expression cassette or a vector according to the invention.

According to the present invention, the plant transformation can be obtained by any suitable known means; plant transformation techniques are fully described in the specialist literature.

Certain techniques use *Agrobacterium* in particular for transforming dicotyledons. A series of methods consists in using a chimeric gene inserted into an *Agrobacterium tumefaciens* Ti plasmid or an *Agrobacterium rhizogenes* Ri plasmid as means of transfer into the plant. Other methods consist in bombarding the cells, protoplasts or tissues with particles to which the DNA sequences are attached. Other methods can also be used, such as microinjection or electroporation, or else direct precipitation with PEG.

Those skilled in the art will soon see appropriate methods as a function of the nature of the plant cell or of the plant.

The present invention relates to the transformed plant cells comprising an expression cassette and/or a vector according to the invention.

According to the invention, the term "plant cell" means any cell derived from a plant and which can constitute undifferentiated tissues such as calluses, differentiated tissues such as embryos, parts of plants, plants or seeds.

The present invention also relates to the transformed plants comprising an expression cassette, a vector and/or transformed cells according to the invention.

According to the invention, the term "plant" means any differentiated multicellular organism capable of photosynthesis, in particular monocotyledons or dicotyledons, more particularly crop plants which may or may not be intended for animal or human food, such as maize, wheat, barley, sorghum, rapeseed, soybean, rice, beetroot, tobacco, cotton, etc.

FIGURES

Figure 1 Figure 1 presents the construct 35S-9-LOX used to transform tobacco. The 9-LOX (*LOX1*) coding sequence was obtained by PCR amplification and then inserted between the cauliflower mosaic virus 35S promoter (35S) and the *Agrobacterium tumefasciens* nopaline synthase terminator (tnos). This vector also comprises the neomycin phosphotransferase (*NPTII*) gene which confers resistance to kanamycin in bacteria and plants. "F" ("sense 35S") denotes the sense primer and "R" ("reverse LOX1") denotes the reverse primer.

Figure 2 Figure 2 is a histogram representing the measurement of LOX specific activity in the tobacco plant stems in nKAT/mg of protein. 46-8 WT and 49-10 WT denote the parental lines. S46-21, S46-26 and S49-18 denote the transgenic lines. In fact, prior to *Ppn* inoculation tests, the LOX specific activity and also the level of corresponding transcripts were analyzed for 12-week-old tobacco plant stems. It is observed that the level of LOX specific activity in the three transgenic lines selected is significantly higher than that measured in the corresponding parental lines.

Figure 3 Figure 3 is a histogram representing the measurement of lesion length in mm. These measurements were made 48 hours (48h) or 72 hours (72h) after inoculation. The numbers in brackets correspond to the independent repetitions carried out. The two lines S46-21 and S46-26, inoculated with *Ppn* 1, exhibit lesion length significantly shorter than those obtained with the same pathogenic agent in the wild-type parent, 46-8 WT. Similarly, the line S49-18 exhibits shorter lesions than those measured in its wild-type parent, 49-10 WT, when these two lines are inoculated with *Ppn* 0. These differences are significant at 48 hours and at 72 hours after inoculation.

EXAMPLES

Example 1: Biological material

Wild-type tobacco plants (*Nicotiana tabacum* L.) of the two near-isogenic lines 46-8 (46-8 WT) and 49-10 (49-10 WT) were used (Helgeson et al., *Phytopath.* 62,1439-1443, 1972). These lines differ from one another by the presence, in the 46-8 WT line, of a locus for resistance to race 0 of *Ppn*. Thus, the 46-8 WT line is resistant to race 0 of *Ppn* and sensitive to race 1 of this pathogenic agent, whereas the 49-10 WT line is sensitive to the two races of *Ppn*.

The seeds of wild-type or transgenic lines are sterilized (Rancé et al., *Plant Cell Report*, 13, 647-651, 1994) and sown on solid MS medium supplemented, in the case of the transgenic lines, with kanamycin (50 $\mu g.ml^{-1}$). After 4 weeks of growth *in vitro* (40% hygrometry, constant temperature 25°C, light 60 $\mu mol.m^{-2}.s^{-1}$: 16h, dark: 8h), the kanamycin-resistant wild-type or transgenic plants are transferred onto vermiculite in a culture room (80% hygrometry, light 125 $\mu mol.m^{-2}.s^{-1}$: 16h, 25°C, dark: 8h, 18°C).

The *Ppn* strains used correspond to the 1156 (race 0) and 1452 (race 1) isolates (Hendrix, J.W. & Apple, J.L., *Tobacco Science* 11, 148-150, 1967). The *Ppn* mycelium is cultured in the dark on a solid synthetic medium (Keen, N.T., *Science* 187, 74-75, 1975).

Example 2: Production of the CaMV 35S promoter-LOX1 coding sequence-nos terminator cassette (p35S-LOX1)

TL-J2 is a 2888 bp complementary DNA corresponding to the tobacco *LOX1* gene induced by pathogenesis. The production of this complementary DNA is described by Véronési et al. (Véronési et al., *Plant Physiol.* 108, 1342, 1995), and its sequence is deposited in GenBank under the accession number X84040. This cDNA was used as a matrix for PCR amplification of the *LOX1* coding sequence.

Primers were synthesized to amplify a 2.6 kb DNA fragment covering positions 49 to 2667 of the cDNA:

- Sense primer = 5'-GTTATCAAACAGTTTAAA**ATG**TTTCTGGAG-3'
- Reverse primer = 5'-TGATTTAAAGTT**CTA**TATTGAC-3'

These primers enable, in addition, the introduction of *Dra*I sites (underlined in the primer sequence) upstream of the translation initiation: and downstream of the stop codon (indicated in bold characters in the primer sequence) of the *LOX1* sequence.

The PCR reaction was carried out in a total volume of 25 µl, containing 50 ng of plasmid pTL-J2, 50 pmol of each of the sense and reverse primers above and 2.5 units of *Pfu* DNA polymerase (Stratagene Cloning Systems), and adjusted to 200 µM of each dNTP and 2 mM $MgCl_2$. After denaturation for 5 min at 94°C, the thermocycler program was made up of 20 cycles, each including denaturation for 1 min at 94°C, hybridization for 1 min at 50°C and extension for 6 min at 72°C, followed by a final step of extension for 40 min at 72°C.

The DNA from this reaction was digested with *Dra*I and separated on 0.8% agarose gel. The 2.6 kb blunt-ended fragment was purified from the gel (QiaEx II kit, Qiagen) and cloned into the *Sma*I site of the vector pIPM0 (Rancé et al., *PNAS* 6554-6559, 1998) between the CaMV 35S promoter and the 3' untranslated region of the *Agrobacterium tumefaciens* nopaline synthase gene (nos terminator). This vector also comprises two copies of the neomycin phosphotransferase (*NPTII*) gene which confers kanamycin resistance in bacteria and plants. The ligation mixture was used to transform competent *Escherichia coli* XL1 Blue bacteria, and kanamycin-resistant colonies were selected and then screened for the presence of LOX sequence using the TL-J2 molecular probe. Positive colonies were

cultured and the corresponding plasmids were purified. The orientation of the LOX1 sequence was examined for each of the plasmids by PCR using the following primers:

- F primer, "sense 35S": 5'–GGCCATGGAGTCAAAGATTC-3' targeting nucleotide 6906-6925 of the CaMV 35S promoter (sequence available in Genbank under the accession number J02048).
- R primer, "reverse *LOX1*": 5'-GCTCTGGCATGAAATTTCG-3' targeting nucleotides 2290-2272 (noncoding strand) of TL-J2.

The amplification reactions were carried out in the volume of 50 µl and comprising 100 ng of test plasmid, 10 pmol of each primer and 1 unit of *Taq* DNA polymerase in a medium adjusted to 200 µM of each dNTP and 1.5 mM $MgCl_2$. The thermocycler program included an initial denaturation step of 5 min at 94°C, and then 40 cycles each consisting of denaturation for 1 min at 94°C, hybridization for 1 min at 65°C and extension for 2 min at 72°C, followed by a final elongation step for 10 min at 72°C. The reaction products were separated on 0.8% agarose gel. A plasmid for which the presence of an amplification product of the expected size (2.8 kb) indicated the sense orientation of the LOX sequence relative to the CaMV 35S promoter in the construct was selected. The LOX sequence and the junctions thereof with the promoter and the terminator were entirely sequenced. The plasmid thus verified is called p35S-*LOX1*. The sequence of the CaMV 35S-*LOX1* construct is described in SEQ ID No. 2.

Example 3: Genetic transformation of tobacco

The plasmid p35S-*LOX1* was mobilized in the *Agrobacterium tumefaciens* strain LBA4404 by heat shock (Holsters et al., *Mol. Gen. Genet.* 163, 181-187, 1978). A kanamycin-resistant colony was isolated, the plasmid was purified, and the entire construct was verified by PCR with the F and R primers and under the conditions described above for determining the relative orientation of the LOX sequence. The recombinant bacteria obtained were then used to infect foliar discs of tobacco, *Nicotiana tabacum*, lines 46-8 WT and 49-10 WT, according to already described protocols (Horsch et al., *Science* 227, 1229-1231, 1985).

The plants regenerated on a Murashige and Skoog (MS) medium containing 150 $\mu g.ml^{-1}$ of kanamycin were placed in a culture chamber and then in a greenhouse so as to obtain T1 seeds, by self-pollination. The transgenic lines regenerated from the parental lines 46-8 WT and 49-10 WT are called S46-x and S49-x plants, respectively.

Example 4: Characterization of the primary transformants

The presence of the 35S-*LOX1* expression cassette and also the number of copies of the transgene in the genome of the regenerated plants were determined by PCR and DNA/DNA (Southern) hybridization experiments. The genomic DNA of wild-type plants or of regenerated plants which were resistant to kanamycin was prepared according to the method described by Dellaporta et al. (Dellaporta et al., *Plant Mol. Biol. Rep.* 1, 19-21, 1983). The entire T-DNA transferred was verified by PCR amplification using the **F** "sense 35S" and **R** "reverse *LOX1*" primers described above. The reaction conditions were those described above, but the amount of matrix DNA was, in this case, 800 ng of genomic DNA. The number of copies of T-DNA inserted was estimated by Southern blotting (50). The genomic DNA (15 μg) was digested with *Bam*H1 and the digestion products were separated on agarose gel and then transferred onto nylon membrane. A CaMV 35S probe (nucleotides 6909 to 7440 of the Genbank sequence J02048) was used after labeling with [α-^{32}P] dCTP. The number of bands hybridizing this probe, after revelation by autoradiography, is a good indication of the number of sites and insertion of the construct.

Example 5: Transformation of tobacco with the sequence encoding tobacco LOX1 under the control of the CaMV 35S constitutive promoter

In order to constitutively express tobacco LOX1 in transgenic tobacco plants, the corresponding coding sequence was introduced in the sense orientation into the transfer DNA of the binary vector pIPM0, downstream of the CaMV 35S constitutive promoter (p35S) (Fig. 1*A*). This construct, called p35S-*LOX1*, also contains a kanamycin resistance gene (*NPTII*) for selecting the transformed plant cells. The 46-8 WT and 49-10 WT tobacco lines were transformed with *Agrobacterium tumefaciens* LBA 4404 into which the p35S-*LOX1* construct has been introduced. 15 independent primary transformants S46-x derived from the 46-8 WT line were regenerated from calluses selected on a medium containing kanamycin. The letter x denotes the number of the plant obtained. Similarly, 25 independent primary transformants S49-x were regenerated from the 49-10 WT line. These plants were acclimatized in a culture chamber and then transferred into a greenhouse until flowering. The seeds corresponding to the T1 plants were obtained by self-pollination of the primary transformants.

The entire construct introduced into the genome of the transgenic plants was verified by PCR amplification using a preparation of genomic DNA of the primary transformants cultured on kanamycin, and a pair of primers, one specific for the 5' region of the CaMV

35S promoter (F) and the other for the 3' region of the *LOX1* coding sequence (R). The amplification products were separated on agarose gel and revealed with ethidium bromide. For 10 of the 11 primary transformants analyzed, the profile obtained corresponds to a single band for which the size (2.8 kb) corresponds to the estimated size of the product. In addition, this profile is identical to that obtained with the binary vector p35S-*LOX1*, which suggests that at least one copy has been integrated into the genome of these transformants. On the other hand, one primary transformant does not have such a profile, although it is resistant to kanamycin, indicating an incomplete integration of the construct. The parental lines 46-8 WT and 49-10 WT, analyzed as negative controls, show no signal corresponding to the construct.

The number of copies inserted into each of the regenerated lines was estimated by Southern-type hybridization using genomic DNA digested with *Bam*HI and a probe homologous to the CaMV 35S promoter. The transfer DNA has two *Bam*HI sites: the first is located between the CaMV 35S promoter and the *LOX1* sequence and a second is located in the *LOX1* sequence. The *Bam*HI fragments which hybridize with the radiolabeled CaMV 35S probe therefore result from a first cleavage between the CaMV 35S promoter and *LOX1* and a second cleavage in the plant genome, upstream of the left border of the transfer DNA. Since the insertion of the transfer DNA into the plant genome is random, the *Bam*HI fragments which hybridize with the radiolabeled CaMV 35S probe, obtained in the case of multiple insertions, will have sizes which depend on the position of the *Bam*H1 site in the genomic DNA and which are therefore probably different. For this reason, the number of these fragments makes it possible to evaluate the number of sites for insertion into the genome. The profiles obtained indicate that the primary transformants S46-3, S46-4, S46-26, S49-8 and S49-13 contain one copy of the transgene, whereas two copies have been inserted into the genome of the lines S49-18 and S49-28, and three copies into the genome of the lines S46-21, S49-14 and S49-30. The radiolabeled CaMV 35S probe did not hybridize with the genomic DNA corresponding to the S49-24 lines, or with that of the parental lines 46-8 WT and 49-10 WT.

Example 6: RNA extraction and analysis

The total RNA was isolated from frozen samples of T1 generation or wild-type plants. The plant material was ground in liquid nitrogen and the RNA was extracted using the Extract-all kit (Eurobio). The nucleic acid concentration was estimated by spectrophotometry. Northern blotting experiments were carried out as previously described

(Rickauer et al., *Planta* 202, 155-162, 1997). The filters were hybridized with the radiolabeled TL-J2 probe.

Example 7: Analysis of LOX transcript accumulation in the T1 transgenic lines

The level of LOX expression was evaluated in the various T1 transgenic lines by measuring the LOX transcript accumulation by Northern blotting. The total RNA samples were prepared from 4-week-old young transgenic tobacco plants selected *in vitro* on a medium containing kanamycin. Evaluation of the respective levels of expression of the transgene in the lines was carried out by comparing the obtained profiles with the level of transcripts detected in wild-type plants, and also in a control tobacco cell suspension (negative control) or in a suspension of tobacco cells treated with *Ppn* elicitors (positive control). The results obtained indicate that the level of transcripts is low, or even undetectable, in the transgenic lines S46-3, S46-4, S49-8, S49-13, S49-24, S49-28 and S49-30. On the other hand, the lines S46-21, S46-26, S49-14 and S49-18 exhibit a considerable accumulation of LOX transcripts, reaching, after quantification, from 30 to 66% of the level detected in the elicited tobacco cells. No LOX transcript accumulation is detected in the wild-type line or in the control tobacco cells. Introduction of the 35S promoter–*LOX1* construct into the tobacco plant is therefore accompanied by considerable constitutive expression in the transgenic lines S46-21, S46-26, S49-14 and S49-18.

Example 8: Immunodetection of LOX

Production of an anti-tobacco LOX1 rabbit polyclonal serum: Rabbits were immunized with a fusion protein expressed in *Escherichia coli* and comprising the 244 C-terminal residues of tobacco LOX1 fused with *Schistosoma japonicum* glutathione S-transferase (GST). An *Xho*I fragment of pTL-J2, corresponding to nucleotides 1921 to 2888, was inserted into the *Xho*I site of the vector pGEX-5X-3 (Pharmacia, sequence available in Genbank under the accession number U13858) which made it possible to obtain a translational fusion with the GST coding sequence. A colony of bacteria containing the recombinant plasmid was selected and placed in culture. These bacteria were treated with 4 mM isopropylthio-β-galactoside for 16 hours at 37°C in order to induce fusion protein production. The bacteria were harvested by centrifugation at 6000 x *g* for 10 min and the proteins were then extracted by resuspension of the bacterial pellet in a buffered solution adjusted to 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3, in a proportion of 40 µl of solution per ml of culture, and then sonication of the mixture in 3

cycles each of 1 min, on ice. The sonicate was centrifuged at 10 000 x *g* for 5 min and the insoluble proteins contained in the centrifugation pellet were recovered and extracted in 1X SDS-PAGE loading buffer (50) at 100°C for 10 min. After further centrifugation at 10 000 x *g* for 5 min, the protein extract was loaded onto an 8% polyacrylamide denaturing gel. After electrophoresis and restaining with Coomassie blue, the gel was destained and the band corresponding to the fusion protein (55 kDa) was excised from the gel and used to immunize the animals (Eurogentec). One of the sera, which exhibited the best titer relative to the fusion protein and to tobacco LOX1 was selected as anti-LOX1 serum. Western blotting analysis: Dialyzed and concentrated enzyme extracts, prepared as described above, were separated by SDS-PAGE on a 10% gel at a rate of 100 µg of protein per lane and, after electrophoresis, the separated proteins were transferred onto nitrocellulose membrane by electroblotting. The Western blotting analyses were carried out according to standard protocols. The anti-LOX1 serum, at a 1:1000 dilution, was used as primary antibody, and anti-rabbit IgG goat IgGs, coupled to alkaline phosphatase (sigma), were used as secondary antibodies. The alkaline phosphatase enzyme activity was detected by the NBT-BCIP method.

Example 9: Detection of the LOX1 protein in the T1 transgenic lines

Western blotting analysis was used to search for the *LOX1* protein in the transgenic lines constitutively expressing the LOX1 transgene. Soluble protein extracts prepared from the aerial parts of 8-week-old plants were separated by SDS-PAGE. The LOX1 protein was detected using a rabbit polyclonal serum directed against the C-terminal portion of the LOX1 protein. Immunochemical revelation shows the presence of a single band in the lanes corresponding to the transgenic lines S46-26 and S49-18. The size of the corresponding product, between 79 and 101 kDa, is coherent with the size calculated from the primary sequence of the LOX1 protein (92 kDa). On the other hand, the LOX1 protein is not detected in the extracts prepared from the parental lines 46-8 WT and 49-10 WT. Constitutive expression of the *LOX1* transgene is therefore accompanied by accumulation of the corresponding protein in the transgenic lines S46-26 and S49-18.

Example 10: Measurement of the LOX activity

The samples of wild-type or transgenic plants were frozen and then ground in liquid nitrogen and homogenized in 0.25 M sodium phosphate buffer, pH 6.5, containing 5% polyvinylpolypyrrolidone, at a rate of 1 ml of buffer per g of fresh material. After thawing,

the extracts were mixed by vortexing and centrifuged for 5 min at 12 000 x *g*. The centrifugation supernatant constitutes the crude enzyme extract. Two methods for measuring the LOX activity were used.

Chromatographic method (TLC): A protocol was adapted from a method described by Caldelari and Farmer (Caldelari, D. & Farmer, E.E., *Phytochemistry* 47, 599-604, 1998). The LOX assay was carried out with an aliquot of the crude enzyme extract corresponding to 50 µg of proteins, in a total volume of 0.4 ml of 0.25 M sodium phosphate buffer, pH 6.5, saturated with air and containing linoleic acid labeled with ^{14}C on the carbon in the 1-position, at a final concentration of 1.2 µM, for 30 min at 30°C. The reaction mixture was then extracted twice with a methanol/chloroform (2:1) mixture and the organic phases were concentrated under a stream of nitrogen. The extracts were separated by thin layer chromatography on silica plates, in an ether/hexane/formic acid (70:30:1) mixture. The radiolabel products derived from metabolization of the linoleic acid and also the remaining substrate were revealed by phosphorimaging. The amount of substrate remaining in each reaction was estimated by comparison with a control reaction without enzyme extract (ImageQuaNT® software)

Spectrophotometric method: The crude enzyme extract was dialyzed and concentrated by centrifugation on an Ultrafree-4 unit (millipore) equipped with a Biomax 10 kDa NMWL membrane, for 30 min at 3500 x *g* and at 4°C, and was then subjected to three washing steps by addition of 0.5 ml of 0.25 M sodium phosphate buffer, pH 6.5 and centrifugation in the same unit. The LOX activity was determined in an assay consisting of a total volume of 475 µl, by measuring the formation of conjugated dienes at λ_{234} nm ($\varepsilon = 27\ 000\ M^{-1}.cm^{-1}$) for 4 min at 30°C, in air-saturated 0.25 M sodium phosphate buffer, pH 6.5. The linoleic acid was used as substrate at a final concentration of 820 µM. The results are expressed in nanokatal.mg^{-1} proteins. The protein content of the aliquots tested was determined by the Bradford method (Bradford, *Anal. Biochem.* 72, 248-254, 1976).

Example 11: *in vitro* conversion of linoleic acid by the T1 transgenic plants

The level of LOX activity of the transgenic plants was compared with that of the parental lines 46-8 WT and 49-10 WT by measing, *in vitro*, the ability of various enzyme extracts to convert a natural substrate of this enzyme, linoleic acid. These extracts, prepared from the aerial parts of 8-week-old plants, were incubated *in vitro* with ^{14}C-labeled linoleic acid. The radiolabeled products, extracted and then separated by thin layer chromatography (TLC), were revealed using a phosphorimager. From the digitized TLC image, the linoleic

acid not metabolized at the end of the reaction was quantified for each lane and expressed as percentage of the linoleic acid measured in a control reaction not containing any enzyme extract. These percentages correspond to the mean of three independent repetitions. In the assays, the linoleic acid disappears almost completely in the lanes corresponding to the transgenic plants S46-26 and S49-18 with only 5 and 10% of substrate remaining at the end of the reaction, whereas, in the case of the parental lines 46-8 WT and 49-10 WT, approximately 50% of the substrate is not metabolized. In order to verify that this difference between the WT and transgenic lines is indeed due to the constitutive expression of the transgene introduced, the same reaction was carried out by preincubating the enzyme extracts with ETYA (5,8,11,14-eicosatetraynoic acid), a LOX-specific inhibitor. In this case, approximately 50% of the linoleic acid is detected at the end of the reaction both for the parental lines and for the transgenic lines. This suggests that all the lines have a LOX-independent activity capable of metabolizing part of the fatty acid introduced. When the enzyme extracts are boiled before being incubated with the substrate, between 80 and 90% of the fatty acid is extracted at the end of the reaction, showing that an enzyme reaction is indeed involved and suggesting either that part of the substrate (between 10 and 20%) is chemically degraded, or that it is not extractable under the conditions used. This experiment therefore shows that the transgenic lines S46-26 and S49-18 exhibit ETYA-sensitive linoleic acid-converting activity, which is not the case of the parental lines 46-8 WT and 49-10 WT. This shows that constitutive expression of the *LOX1* transgene, and also the presence of the LOX1 protein in the transgenic lines, also results in an increase in LOX activity in these plants. This increase in activity was also measured in the S46-21 line.

Example 12: Inoculation of tobacco plants with *Ppn*

A method of stem inoculation of tobacco with *Ppn* was used. 12-week-old wild-type (46-8 WT and 49-10 WT lines) or transgenic tobacco plants were inoculated by application of a disc of mycelium onto the stem after sectioning of the apical part thereof (approximately a third of the way from the top) with a razor blade. The discs of mycelium originated from 7-day-old cultures in agar medium. Control plants were treated identically, with the exception of the application of the mycelium disc, replaced with a disc of sterile medium. The control and inoculated stems were covered with an aluminum film so as to preserve the plant tissues and the mycelium against desiccation.

Example 13: Observation and measurement of symptoms

The symptoms were observed and quantified 48 hours or 72 hours after inoculation. The stems were sectioned longitudinally and the length of the lesions was measured for each half-stem at five equidistant points, distributed over the entire width of the section. The lesion length used for each individual corresponds to the mean of these 10 measurements.

Example 14: Measurement of *LOX1* transcript accumulation and of LOX specific activity in the stems of the T1 transgenic lines

The method selected for testing the interaction between tobacco and the pathogenic microorganism *Ppn* consists in inoculating the stem with *Ppn* mycelium, after sectioning the apex of the plant. As a prelude to this experiment, the level of expression of the transgene and also the LOX specific activity in the stems of the transgenic lines S46-21, S46-26 and S49-18 were compared with those observed in the parental lines 46-8 WT and 49-10 WT. For each line, total RNAs were prepared from a pool of 3 pieces of stem each originating from an independent plant. The result of hybridization with a radiolabeled *LOX1* probe confirms LOX transcript accumulation in the stems of the transgenic lines S46-21, S46-26 and S49-18, whereas no LOX transcript is detected in the parental lines 46-8 WT and 49-10 WT. The LOX specific activity was also measured in this organ, using concentrated and dialyzed enzyme extracts. The analysis was carried out in a spectrophotometer by measuring the appearance of fatty acid hydroperoxides at 234 nm. For each line studied, 3 independent measurements were taken. The results obtained, pooled in a histogram (Fig. 2), indicate that the LOX specific activity measured in the transgenic lines S46-21, S46-26 and S49-18 is 1.8 to 5 times greater than the level of activity measured in the parental lines 46-8 WT and 49-10 WT. In addition, these levels of activity reach 25% (S46-21) and 70% (S49-18) of the level of LOX activity measured in tobacco cells elicited for 24 hours (353.8 $nkat.mg^{-1}$ protein, data not shown). This analysis therefore confirms that the constitutive expression of the LOX transgene, and also the increase in LOX activity, measured in the transgenic plants also characterizes the stems.

Example 15: Analysis of the interaction between *Ppn* and the T1 transgenic lines having constitutive LOX activity

In order to examine the consequences of constitutive *LOX1* expression in the transgenic lines S46-21, S46-26 and S49-18 on their interaction with *Ppn*, 12-week-old plants of these lines were inoculated with this pathogenic agent at the level of the stems. The

symptoms obtained after inoculation with a virulent race of *Ppn* were compared with those observed during a compatible interaction involving the corresponding parental line. Thus, the lines S46-21, S46-26 and 46-8 WT were inoculated with race 1 of *Ppn*, whereas the lines S49-18 and 49-10 WT were inoculated with race 0 of *Ppn*. An incompatibility control was carried out by inoculating the line 46-8 WT with race 0 of *Ppn*. The symptoms obtained 48 hours or 72 hours after inoculation were observed on longitudinal sections of the stems and the lesions were measured (Figure 3).

The symptoms observed on the parental lines 46-8 WT and 49-10 WT are typical of tobacco/*Ppn* interactions; the line 46-8 WT, inoculated with race 0 of *Ppn*, exhibits dry and localized lesions characteristic of an incompatible interaction. On the other hand, the long brown macerated lesions observed in the 46-8 WT/*Ppn* 1 and 49-10 WT/*Ppn* 0 interactions reflect the colonization of the stem by the pathogenic agent and are typical of compatible interactions. In comparison with the latter, the lesions measured in the transgenic lines, inoculated with the same virulent race as that used with the corresponding parental line, are clearly reduced. In the two transgenic lines selected, S46-21 and S46-26, inoculation with race 1 of the fungus does not cause the formation of these long macerated lesions. Lesions are observed which are much more reduced than in the compatible case, but also much less macerated. This difference is also observed when the compatible interaction 49-10 WT/*Ppn* 0 (*Ppn* 0-sensitive wild-type line) and the interaction between the transgenic line S49-18, which is derived from the line 49-10 WT, and *Ppn* 0 are compared. It is observed that the lesions caused during the S46-26/*Ppn* 1 interaction resemble more closely the necroses which appear during an incompatible interaction (46-8 WT/*Ppn* 0) than the lesions which accompany the colonization of the plant tissues by the fungus in the case of a compatible interaction (46-8 WT/*Ppn* 1). For example, the lesions obtained 48 hours after inoculation in the S46-21/*Ppn* 1 and S46-26/*Ppn* 1 interactions are respectively 3.4 and 2.4 times shorter than those measured in the compatible interaction 46-8 WT/*Ppn* 1. For the S49-18 line, inoculation with race 0 of *Ppn* causes lesions twice as short as those observed for the parental line 49-10 WT inoculated with the same race of the fungus. All these results show that constitutive LOX expression in the transgenic lines is accompanied by a clear limitation of the progression of the fungus.

Besides the reduction in size of the lesions observed in the transgenic lines inoculated with a virulent race of *Ppn*, the nature thereof is also modified. The lesions obtained in the S46-26/*Ppn* 1 interaction are not macerated as in the compatible interaction 46-8 WT/*Ppn* 1, but are rather dry as in the incompatible interaction 46-8 WT/*Ppn* 0. This

shows that the constitutive LOX activity measured in the transgenic lines S46-21, S46-26 and S49-18 actively contribute to the resistance of tobacco to *Ppn*.

Example 16: Analysis of the interaction between Ppn and the T1 transgenic plants having constitutive LOX activity – root inoculation

Plants constitutively expressing tobacco LOX1 were previously obtained. In a stem inoculation test, the plants showed reduced sensitivity to *Phytophthora parasitica* var. *nicotianae* (*Ppn*) in comparison with the wild-type line from which they are derived. The behavior of these plants was studied in a **root inoculation** test.

Wild-type tobacco plants (*Nicotiana tabacum* L.) of the 46-8 line (46-8 WT), characterized by the presence of a locus for resistance to race 0 of *Ppn*, and plants of the sense-lipoxygenase S46-21 transgenic line, derived from the 46-8 WT line, were sown *in vitro*. The seeds of wild-type or transgenic lines were sterilized and sown in Petri dishes, on solid MS medium at a rate of approximately 30 seeds per dish, by intercollating a disc of synthetic cloth between the medium and the seeds. The dishes were placed in a sloping position so as to orient the growth of the roots. After 3 weeks of growth *in vitro* (40% hygrometry, constant temperature 25°C, light 60 $\mu mol.m^{-2}.s^{-1}$: 16h, dark: 8h), the discs of cloth supporting the plants were transferred into new Petri dishes containing liquid MS medium and glass beads, and the plants were returned to the culture chamber for 2 days, before inoculation. The *Ppn* strains used correspond to isolates 1156 (race 0) and 1452 (race 1). The *Ppn* mycelium was grown in the dark on a solid synthetic medium.

A method for root inoculation of tobacco using a suspension of *Ppn* zoospores was used. A *Ppn* mycelium colony obtained on V8 medium was placed under conditions of deficiency on agar-water for 4 days, and the zoospores were then released by cold shock (30 min at 15°C and then 30 min at ambient temperature) in 10 ml of water. After counting, the zoospore suspension is adjusted to 4000 spores/ml. For each dish, the liquid MS medium is removed and replaced with the spore suspension. The plants exhibiting no symptoms of disease are counted after 6 to 11 days, and the percentage survival (plants free of symptoms/total plants) is calculated for each combination.

In order to examine the consequences of constitutive *LOX1* expression in tobacco on the interaction with *Ppn*, 3-week-old plants of the S46-21 transgenic line were inoculated at the level of the roots with virulent race 1 of this pathogenic antigen. The outcome of the inoculated plants was compared with that of wild-type plants of the corresponding parental line 46-8 WT, inoculated with this same race (compatible interaction). An incompatibility

control was carried out, by inoculating the 46-8 WT line with race 0 of *Ppn* under the same conditions, as was a noninoculated control. The plants were observed 6 or 11 days after inoculation in a first experiment, and 7 days after inoculation in a second experiment. The plants free of symptoms of disease were counted and the percentage survivals were calculated (Table 1).

The 46-8 WT line inoculated with race 0 of *Ppn* exhibits no symptoms and the percentage survival is very close to 100%. On the other hand, in the 46-8 WT/*Ppn* 1 interaction, the colonization of the plants by the pathogenic antigen results in considerable mortality and a low percentage survival (20 to 24%). In comparison with this compatible interaction, the plants of the sense LOX S46-21 transgenic line, inoculated with the same virulent race as that used with the 46-8 WT line of origin, exhibit a much higher percentage survival, in two independent experiments (survival rate 80 to 88%). All these results confirm that constitutive LOX expression tobacco is accompanied by a notable reduction in sensitivity to *Ppn*.

Table 1. Percentage survival of the wild-type or sense-LOX transgenic plants, after inoculation with virulent race 1 of *Ppn*, and comparison with the incompatible interaction 46-8 WT/race 0

	Total number of plants	Survival rate[a]	
EXPERIMENT N° 1		D6	D11
46-8 WT / *Ppn* 0 **(incompatible)**	n=58	100%	100%
46-8 WT / *Ppn* 1 **(compatible)**	n=55	24.0%	20.0%
S46-21 / *Ppn* 1	n=54	88.8%	88.8%
EXPERIMENT N° 2		D7	
46-8 WT **control**	n=47	95.7%	
46-8 WT / *Ppn* 1 **(compatible)**	n=82	21.9%	
S46-21 / *Ppn* 1	n=65	80.0%	

[a] Survival rate = % number of plants without symptoms of disease/total number of plants.

Bibliographic references

1. **Brash, A. R., Ingram, C. D. & Harris, T. M.** (1987) Analysis of a specific oxygenation reaction of soybean lipoxygenase-1 with fatty acids esterified in phospholipids, *Biochemistry* 26, 5465-5471.

2. **Maccarrone, M., van Aarle, P. G., Veldink, G. A. & Vliegenthart, J. F.** (1994) *In vitro* oxygenation of soybean biomembranes by lipoxygenase-2, *Biochim. Biophys. Acta* 1190, 164-169.

3. **Feussner, I., Bachmann, A., Hohne, M. & Kindl, H.** (1998) All three acyl moieties of trilinolein are efficiently oxygenated by recombinant His-tagged lipid body lipoxygenase *in vitro*, *FEBS Lett.* 431, 433-436.

4. **Blée, E.** (1998) Phytooxylipins and plant defense reactions, *Prog. Lipid Res.* 37, 33-72.

5. **Rosahl, S.** (1996) Lipoxygenases in plants - their role in development and stress response, *Z. Naturforsch., C: Biosci.* 51, 123-138.

6. **Hornung, E., Walther, M., Kuhn, H. & Feussner, I.** (1999) Conversion of cucumber linoleate 13-lipoxygenase to a 9-lipoxygenating species by site-directed mutagenesis, *Proc. Natl. Acad. Sci. U. S. A.* 96, 4192-4197.

7. **Kuhn, H. & Thiele, B. J.** (1999) The diversity of the lipoxygenase family. Many sequence data but little information on biological significance, *FEBS Lett* 449, 7-11.

8. **Galliard, T. & Chan, H. W. S.** (1980) in *The Biochemistry of Plants A comprehensive treatise*, ed. Stumpf, P. K. (Academic Press, INC, New York), Vol. 4, pp. 131-161.

9. **Slusarenko, A. J.** (1996) in *Lipoxygenase and lipoxygenase pathway enzymes*, ed. Piazza, G. J. (AOCS Press, Champain, Illinois), pp. 176-197.

10. **Croft, K. P. C., Jüttner, F. & Slusarenko, A. J.** (1993) Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv. *phaseolicola*, *Plant Physiol.* 101, 13-24.

11. **Bate, N. J. & Rothstein, S. J.** (1998) C6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes, *Plant J.* 16, 561-569.

12. **Reymond, P. & Farmer, E. E.** (1998) Jasmonate and salicylate as global signals for defense gene expression, *Current Opinion in Plant Biology* 1, 404-411.

13. **McConn, M., Creelman, R. A., Bell, E., Mullet, J. E. & Browse, J.** (1997) Jasmonate is essential for insect defense in *Arabidopsis*, *Proc. Natl. Acad. Sci. U. S. A.* 94, 5473-5477.

14. **Staswick, P. E., Yuen, G. Y. & Lehman, C. C.** (1998) Jasmonate signaling mutants of *Arabidopsis* are susceptible to the soil fungus *Pythium irregulare*, *Plant J.* 15, 747-754.

15. **Howe, G. A., Lightner, J., Browse, J. & Ryan, C. A.** (1996) An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack, *Plant Cell* 8, 2067-2077.

16. **Itoh, A. & Howe, G. A.** (2001) Molecular cloning of a divinyl ether synthase : Identification as a CYP74 cytochrome P-450, *J. Biol. Chem.* 276, 3620-3627.

17. **Weber, H., Chetelat, A., Caldelari, D. & Farmer, E. E.** (1999) Divinyl ether fatty acid synthesis in late blight-diseased potato leaves, *Plant Cell* 11, 485-494.

18. **Schweizer, P., Felix, G., Buchala, A., Muller, C. & Metraux, J. P.** (1996) Perception of free cutin monomers by plant cells, *Plant J.* 10, 331-341.

19. **Rickauer, M., Fournier, J., Pouénat, M. L., Berthalon, E., Bottin, A. & Esquerre-Tugaye, M. T.** (1990) Early changes in ethylene synthesis and lipoxygenase activity during defense induction in tobacco cells, *Plant Physiology and Biochemistry* 28, 647-653.

20. **Fournier, J., Pouénat, M. L., Rickauer, M., Rabinovitch-Chable, H., Rigaud, M. & Esquerré-Tugayé, M. T.** (1993) Purification and characterisation of elicitor-induced lipoxygenase in tobacco cells, *Plant J.* 3, 63-70.

21. **Véronési, C., Fournier, J., Rickauer, M., Marolda, M. & Esquerré-Tugayé, M. T.** (1995) Nucleotide sequence of an elicitor-induced tobacco lipoxygenase cDNA (Genbank X84040), *Plant Physiol.* 108, 1342.

22. **Véronési, C., Rickauer, M., Fournier, J., Pouénat, M. L. & Esquerré-Tugayé, M. T.** (1996) Lipoxygenase gene expression in the tobacco-*Phytophthora parasitica nicotianae* interaction, *Plant Physiol.* 112, 997-1004.

23. **Koch, E., Meier, B. M., Eiben, H. G. & Slusarenko, A. J.** (1992) A lipoxygenase from leaves of tomato (*Lycopersicum esculentum* Mill) is induced in response to plant pathogen *Pseudomonas*, *Plant Physiol.* 99, 571-576.

24. **Peng, Y. L., Shirano, Y., Ohta, H., Hibino, T., Tanaka, K. & Shibata, D.** (1994) A novel lipoxygenase from rice. Primary structure and specific expression upon incompatible infection with rice blast fungus, *J. Biol. Chem.* 269, 3755-3761.

25. **Kolomiets, M. V., Chen, H., Gladon, R. J., Braun, E. J. & Hannapel, D. J.** (2000) A leaf lipoxygenase of potato induced specifically by pathogen infection, *Plant Physiol.* 124, 1121-1130.

26. **Véronési, C.** (1995) (Université Paul Sabatier - Toulouse III, Toulouse), pp. 90.

27. **Villalba Mateos, F., Rickauer, M. & Esquerré-Tugayé, M. T.** (1997) Cloning and characterization of a cDNA encoding an elicitor of *Phytophthora parasitica* var. *nicotianae* that shows cellulose-binding and lectin-like activities, *Mol. Plant-Microbe Interact.* 10, 1045-1053.

28. **Mercier, L., Lafitte, C., Borderies, G., Briand, X., Esquerre-Tugaye, M. T. & Fournier, J.** (2001) The algal polysaccharide carrageenans can act as an elicitor of plant defence, *The New Phytologist* 149, 43-51.

29. **Rickauer, M., Brodschelm, W., Bottin, A., Véronési, C., Grimal, H. & Esquerré-Tugayé, M. T.** (1997) The jasmonate pathway is involved differentially in the regulation of different defence responses in tobacco cells, *Planta* 202, 155-162.

30. **Rancé, I.** (1997) (Université Paris XI - Orsay), pp. 111.

31. **Rancé, I., Fournier, J. & Esquerré-Tugayé, M. T.** (1998) The incompatible interaction between *Phytophthora parasitica* var. *nicotianae* race 0 and tobacco is suppressed in transgenic plants expressing antisense lipoxygenase sequences, *Proc. Natl. Acad. Sci. U. S. A.* 95, 6554-6559.

32. **Rustérucci, C., Montillet, J. L., Agnel, J. P., Battesti, C., Alonso, B., Knoll, A., Bessoule, J. J., Etienne, P., Suty, L., Blein, J. P. & Triantaphylides, C.** (1999) Involvement of lipoxygenase-dependent production of fatty acid hydroperoxides in the

development of the hypersensitive cell death induced by cryptogein on tobacco leaves, *J. Biol. Chem.* 274, 36446-36455.

33. **Göbel, C., Feussner, I., Schmidt, A., Scheel, D., Sanchez-Serrano, J., Hamberg, M. & Rosahl, S.** (2001) Oxylipin profiling reveals the preferential stimulation of the 9-lipoxygenase pathway in elicitor-treated potato cells, *J. Biol. Chem.* 276, 6267-6273.

34. **Royo, J., Leon, J., Vancanneyt, G., Albar, J. P., Rosahl, S., Ortego, F., Castanera, P. & Sanchez-Serrano, J. J.** (1999) Antisense-mediated depletion of a potato lipoxygenase reduces wound induction of proteinase inhibitors and increases weight gain of insect pests, *Proc. Natl. Acad. Sci. U. S. A.* 96, 1146-1151.

35. **Deng, W., Grayburn, W. S., Hamilton-Kemp, T. R., Collins, G. B. & Hildebrand, D. F.** (1992) Expression of soybean-embryo lipoxygenase 2 in transgenic tobacco tissue, *Planta* 187, 203-208.

36. **Hause, B., Weichert, H., Hohne, M., Kindl, H. & Feussner, I.** (2000) Expression of cucumber lipid-body lipoxygenase in transgenic tobacco: lipid-body lipoxygenase is correctly targeted to seed lipid bodies, *Planta* 210, 708-714.

37. **Bell, E., Creelman, R. A. & Mullet, J. E.** (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*, *Proc. Natl. Acad. Sci. U. S. A.* 92, 8675-8679.

38. **Maccarrone, M., Hilbers, M. P., Veldink, G. A., Vliegenthart, J. F. & Finazzi Agro, A.** (1995) Inhibition of lipoxygenase in lentil protoplasts by expression of antisense RNA, *Biochim. Biophys. Acta* 1259, 1-3.

39. **Grechkin, A.** (1998) Recent developments in biochemistry of the plant lipoxygenase pathway, *Prog. Lipid Res.* 37, 317-352.

40. **Laudert, D., Pfannschmidt, U., Lottspeich, F., Hollander-Czytko, H. & Weiler, E. W.** (1996) Cloning, molecular and functional characterization of *Arabidopsis thaliana* allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates, *Plant Mol. Biol.* 31, 323-335.

41. **Bate, N. J., Sivasankar, S., Moxon, C., Riley, J. M., Thompson, J. E. & Rothstein, S. J.** (1998) Molecular characterization of an *Arabidopsis* gene encoding hydroperoxide lyase, a cytochrome P-450 that is wound inducible, *Plant Physiol.* 117, 1393-1400.

42. **Howe, G. A., Lee, G. I., Itoh, A., Li, L. & DeRocher, A. E.** (2000) Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase, *Plant Physiol* 123, 711-24.

43. **Helgeson, J. P., Kemp, J. D., Haberlach, G. T. & Maxwell, D. P.** (1972) A tissue culture system for studying disease resistance : the black shank disease in tobacco callus culture, *Phytopath.* 62, 1439-1443.

44. **Rancé, I., Tian, W. Z., Mathews, H., De Kochko, A., Beachy, R. N. & Fauquet, C. M.** (1994) Partial dessication of mature embryo-derived calli, a simple treatment that dramatically enhances the regeneration ability of indica rice., *Plant Cell Report* 13, 647-651.

45. **Hendrix, J. W. & Apple, J. L.** (1967) Stem resistance to *Phytophthora parasitica* var. *nicotianae* in tobacco derived from *Nicotiana longiflora* and *Nicotiana plumbaginifolia*, *Tobacco Science* 11, 148-150.

46. **Keen, N. T.** (1975) Specific elicitors of plant phytoalexin production : determinants of race specificity in pathogens?, *Science* 187, 74-75.

47. **Holsters, M., de Waele, D., Depicker, A., Messens, E., van Montagu, M. & Schell, J.** (1978) Transfection and transformation of *Agrobacterium tumefaciens*, *Mol. Gen. Genet.* 163, 181-187.

48. **Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eicholtz, D., Rogers, S. G. & Fraley, R. T.** (1985) A simple and general method for transferring genes into plants, *Science* 227, 1229-1231.

49. **Dellaporta, S. L., Wood, J. & Hicks, J. B.** (1983) A plant DNA minipreparation : version II., *Plant Mol. Biol. Rep.* 1, 19-21.

50. **Sambrook, J., Fritsh, E. F. & Maniatis, T.** (1989) *Molecular cloning : a laboratory manual* (Cold Spring Harbor Laboratory Press, New-York).

51. **Caldelari, D. & Farmer, E. E.** (1998) A rapid assay for the coupled cell free generation of oxylipins, *Phytochemistry* 47, 599-604.

52. **Bradford, M. M.** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72, 248-254.

53. **Seo, H. S., Song, J. T., Cheong, J.-J., Lee, Y.-H., Lee, Y.-W., Hwang, I., Lee, J. S. & Choi, Y. D.** (2001) Jasmonic acid carboxyl methyltransferase: A key enzyme for jasmonate-regulated plant responses, *Proc. Natl. Acad. Sci. U. S. A.* 98, 4788-4793.

Claims

1) A method for reducing plant sensitivity to diseases and attacks from pathogenic organisms, characterized in that it consists in overexpressing a lipoxygenase in said plants.

2) The method as claimed in claim 1, characterized in that it consists in constitutively overexpressing a lipoxygenase in said plants.

3) The method as claimed in either of claims 1 and 2, in which said lipoxygenase has 9-lipoxygenase activity.

4) The method as claimed in one of claims 1 to 3, in which said lipoxygenase is a plant lipoxygenase.

5) The method as claimed in one of claims 1 to 4, in which said lipoxygenase is a Solanacea plant lipoxygenase.

6) The method as claimed in one of claims 1 to 5, in which said lipoxygenase is at least 80% homologous to the lipoxygenase of SEQ ID No. 1.

7) The method as claimed in claim 6, in which said lipoxygenase is represented in SEQ ID No. 1.

8) The method as claimed in one of claims 1 to 7, in which said lipoxygenase is overexpressed by integration into the genome of said plant of an expression cassette comprising a sequence encoding said lipoxygenase under the control of a promoter which is functional in plants.

9) The method as claimed in claim 8, in which said promoter is a promoter which is constitutive in plants.

10) The method as claimed in claim 9, in which said constitutive promoter is the cauliflower mosaic virus 35S promoter.

11) The method as claimed in one of claims 1 to 10, in which said lipoxygenase is overexpressed in the stems, the leaves and the roots of said plants.

12) An expression cassette which is functional in plant cells and plants, characterized in that it comprises a promoter having constitutive activity in plants, controlling the expression of a polynucleotide encoding a lipoxygenase at least 90% homologous to lipoxygenase of SEQ ID No. 1.

13) The expression cassette as claimed in claim 12, in which said polynucleotide encodes a lipoxygenase having 9-lipoxygenase activity.

14) The expression cassette as claimed in either of claims 12 and 13, in which said polynucleotide encodes the lipoxygenase of SEQ ID No. 1.

15) The expression cassette as claimed in one of claims 12 to 14, in which said promoter is the cauliflower mosaic virus 35S promoter.

16) A vector, characterized in that it comprises an expression cassette as claimed in one of claims 12 to 15.

17) A transformed plant cell, characterized in that it comprises an expression cassette as claimed in one of claims 12 to 15 and/or a vector as claimed in claim 16.

18) A transformed plant, characterized in that it comprises an expression cassette as claimed in one of claims 12 to 15, a vector as claimed in claim 16 and/or transformed plant cells as claimed in claim 17.

Abstract

The present invention relates to methods for reducing plant sensitivity to diseases and attacks from pathogenic organisms. The methods according to the invention consist in overexpressing an inducible lipoxygenase in plants in order to improve their response to diseases and pathogens. The invention also relates to expression cassettes for overexpressing lipoxygenases in plants, and also to transformed plants.

SEQUENCE LISTING

<110> RHOBIO

<120>

<130> PM01021 G1

<140>
<141>

<150> FR 01 07470
<151> 2001-06-07

<160> 6

<170> PatentIn Ver. 2.1

<210> 1
<211> 862
<212> PRT
<213> Nicotiana tabacum

<300>
<308> X84040

<400> 1

```
Met Phe Leu Glu Lys Ile Val Asp Ala Ile Thr Gly Lys Asp Asp Gly
  1               5                  10                  15

Lys Lys Val Lys Gly Thr Val Val Leu Met Lys Lys Asn Val Leu Asp
             20                  25                  30

Phe Thr Asp Ile Asn Ala Ser Val Leu Asp Gly Val Leu Glu Phe Leu
         35                  40                  45

Gly Arg Arg Val Ser Leu Glu Leu Ile Ser Ser Val Asn Ala Asp Pro
     50                  55                  60

Ala Asn Gly Leu Gln Gly Lys Arg Ser Lys Ala Ala Tyr Leu Glu Asn
 65                  70                  75                  80

Trp Leu Thr Asn Ser Thr Pro Ile Ala Ala Gly Glu Ser Ala Phe Arg
                 85                  90                  95

Val Thr Phe Asp Trp Asp Asp Glu Glu Phe Gly Val Pro Gly Ala Phe
            100                 105                 110

Ile Ile Lys Asn Leu His Phe Ser Glu Phe Phe Leu Lys Ser Leu Thr
        115                 120                 125

Leu Glu Asp Val Pro Asn His Gly Lys Val His Phe Val Cys Asn Ser
    130                 135                 140

Trp Val Tyr Pro Ala Asn Lys Tyr Lys Ser Asp Arg Ile Phe Phe Ala
145                 150                 155                 160

Asn Gln Ala Tyr Leu Pro Ser Glu Thr Pro Asp Thr Leu Arg Lys Tyr
                165                 170                 175

Arg Glu Asn Glu Leu Val Thr Leu Arg Gly Asp Gly Thr Gly Lys Leu
            180                 185                 190
```

```
Glu Glu Trp Asp Arg Val Tyr Asp Tyr Ala Tyr Tyr Asn Asp Leu Gly
        195                 200                 205

Asp Pro Asp Lys Gly Gln Asp Leu Ser Arg Pro Val Leu Gly Gly Ser
    210                 215                 220

Ser Glu Tyr Pro Tyr Pro Arg Arg Gly Arg Thr Gly Arg Lys Pro Thr
225                 230                 235                 240

Lys Thr Asp Pro Asn Ser Glu Ser Arg Ile Pro Leu Leu Met Ser Leu
                245                 250                 255

Asp Ile Tyr Val Pro Arg Asp Glu Arg Phe Gly His Ile Lys Leu Ser
            260                 265                 270

Asp Phe Leu Thr Phe Ala Leu Lys Ser Ile Val Gln Leu Leu Leu Pro
        275                 280                 285

Glu Phe Lys Ala Leu Phe Asp Ser Thr His Asn Glu Phe Asp Ser Phe
    290                 295                 300

Glu Asp Val Leu Lys Leu Tyr Glu Gly Gly Ile Lys Leu Pro Gln Gly
305                 310                 315                 320

Pro Leu Leu Lys Ala Ile Thr Asp Ser Ile Pro Leu Glu Ile Leu Lys
                325                 330                 335

Glu Leu Leu Arg Ser Asp Gly Glu Gly Leu Phe Lys Tyr Pro Thr Pro
            340                 345                 350

Gln Val Ile Gln Glu Asp Lys Thr Ala Trp Arg Thr Asp Glu Glu Phe
        355                 360                 365

Gly Arg Glu Met Leu Ala Gly Val Asn Pro Val Ile Ile Ser Arg Leu
    370                 375                 380

Gln Glu Phe Pro Pro Lys Ser Lys Leu Asp Pro Lys Ile Tyr Gly Asn
385                 390                 395                 400

Gln Asn Ser Thr Ile Thr Arg Glu Gln Ile Glu Asp Lys Leu Asp Gly
                405                 410                 415

Leu Thr Ile Asp Glu Ala Ile Lys Thr Asn Arg Leu Phe Ile Leu Asn
            420                 425                 430

His His Asp Ile Leu Met Pro Tyr Leu Arg Arg Ile Asn Thr Ser Thr
        435                 440                 445

Asp Thr Lys Thr Tyr Ala Ser Arg Thr Leu Leu Phe Leu Gln Asp Asn
    450                 455                 460

Gly Thr Leu Lys Pro Ser Ala Ile Glu Leu Ser Leu Pro His Pro Asp
465                 470                 475                 480

Gly Asp Gln Phe Gly Ala Val Ser Lys Val Tyr Thr Pro Ala Asp Gln
                485                 490                 495

Gly Val Glu Gly Ser Ile Trp Gln Leu Ala Lys Ala Tyr Ala Ala Val
            500                 505                 510

Asn Asp Ser Gly Val His Gln Leu Ile Ser His Trp Leu Asn Thr His
        515                 520                 525
```

```
Ala Ala Ile Glu Pro Phe Val Ile Ala Thr Asn Arg Gln Leu Ser Ala
    530                 535                 540

Leu His Pro Ile Tyr Lys Leu Leu His Pro His Phe Arg Glu Thr Met
545                 550                 555                 560

Asn Ile Asn Ala Leu Ala Arg Gln Ile Leu Ile Asn Gly Gly Gly Leu
                565                 570                 575

Leu Glu Leu Thr Val Phe Pro Ala Lys Tyr Ser Met Glu Met Ser Ala
            580                 585                 590

Val Val Tyr Lys Asp Trp Val Phe Pro Glu Gln Ala Leu Pro Thr Asp
        595                 600                 605

Leu Ile Lys Arg Gly Val Ala Val Glu Asp Ser Ser Ser Pro Leu Gly
    610                 615                 620

Ile Arg Leu Leu Ile Gln Asp Tyr Pro Tyr Ala Val Asp Gly Leu Lys
625                 630                 635                 640

Ile Trp Ser Ala Ile Lys Ser Trp Val Thr Glu Tyr Cys Asn Tyr Tyr
                645                 650                 655

Tyr Lys Ser Asp Asp Ala Val Gln Lys Asp Thr Glu Leu Gln Ala Trp
            660                 665                 670

Trp Lys Glu Leu Arg Glu Glu Gly His Gly Asp Lys Lys Asp Glu Pro
        675                 680                 685

Trp Trp Pro Lys Met Gln Thr Val Gln Glu Leu Ile Asp Ser Cys Thr
    690                 695                 700

Ile Thr Ile Trp Ile Ala Ser Ala Leu His Ala Ala Val Asn Phe Gly
705                 710                 715                 720

Gln Tyr Pro Tyr Ala Gly Tyr Leu Pro Asn Arg Pro Thr Leu Ser Arg
                725                 730                 735

Asn Phe Met Pro Glu Pro Gly Ser Pro Glu Tyr Glu Glu Leu Lys Thr
            740                 745                 750

Asn Pro Asp Lys Val Phe Leu Lys Thr Ile Thr Pro Gln Leu Gln Thr
        755                 760                 765

Leu Leu Gly Ile Ser Leu Ile Glu Ile Leu Ser Arg His Ser Ser Asp
    770                 775                 780

Thr Leu Tyr Leu Gly Gln Arg Glu Ser Pro Glu Trp Thr Lys Asp Gln
785                 790                 795                 800

Glu Pro Leu Ser Ala Phe Ala Arg Phe Gly Lys Lys Leu Ser Asp Ile
                805                 810                 815

Glu Asp Gln Ile Met Gln Met Asn Val Asp Glu Lys Trp Lys Asn Arg
            820                 825                 830

Ser Gly Pro Val Lys Val Pro Tyr Thr Leu Leu Phe Pro Thr Ser Glu
        835                 840                 845

Gly Gly Leu Thr Gly Lys Gly Ile Pro Asn Ser Val Ser Ile
    850                 855                 860
```

<210> 2
<211> 3390
<212> DNA
<213> Artificial sequence

<220>
<223> Artificial sequence description:
construct CaMV 35S-LOX

<220>
<221> promoter
<222> (1)..(532)
<223> CaMV 35S promoter

<220>
<221> CDS
<222> (543)..(3131)
<223> Tobacco LOX1 coding sequence

<220>
<221> terminator
<222> (3138)..(3390)
<223> Nos terminator

<400> 2
ccatggagtc aaagattcaa atagaggacc taacagaact cgccgtaaag actggcgaac 60

agttcataca gagtctctta cgactcaatg acaagaagaa aatcttcgtc aacatggtgg 120

agcacgacac gcttgtctac tccaaaaata tcaaagatac agtctcagaa gaccaaaggg 180

caattgagac ttttcaacaa agggtaatat ccggaaacct cctcggattc cattgcccag 240

ctatctgtca ctttattgtg aagatagtgg aaaaggaagg tggctcctac aaatgccatc 300

attgcgataa aggaaaggcc atcgttgaag atgcctctgc cgacagtggt cccaaagatg 360

gacccccacc cacgaggagc atcgtggaaa aagaagacct tccaaccacg tcttcaaagc 420

aagtggattg atgtgatatc tccactgacg taagggatga cgcacaatcc cactatcctt 480

cgcaagaccc ttcctcctat aaggaagttc atttcatttg gagaggacac gcggtaccca 540

aa atg ttt ctg gag aag att gtg gat gca atc aca ggg aaa gat gat 587
Met Phe Leu Glu Lys Ile Val Asp Ala Ile Thr Gly Lys Asp Asp
1 5 10 15

gga aaa aag gta aaa gga aca gtg gtt ttg atg aag aaa aat gtt ttg 635
Gly Lys Lys Val Lys Gly Thr Val Val Leu Met Lys Lys Asn Val Leu
20 25 30

gat ttt act gat att aat gcc tca gtt ctt gat gga gtt ctt gag ttc 683
Asp Phe Thr Asp Ile Asn Ala Ser Val Leu Asp Gly Val Leu Glu Phe
35 40 45

ctt ggt cgg agg gtc tct ctc gag ttg atc agt tct gtt aat gct gat 731
Leu Gly Arg Arg Val Ser Leu Glu Leu Ile Ser Ser Val Asn Ala Asp
50 55 60

cct gca aat ggt tta caa ggg aaa cgc agc aaa gca gca tat ttg gag 779
Pro Ala Asn Gly Leu Gln Gly Lys Arg Ser Lys Ala Ala Tyr Leu Glu
65 70 75

```
aac tgg cta aca aat agc acc cca ata gca gca ggt gaa tca gca ttt   827
Asn Trp Leu Thr Asn Ser Thr Pro Ile Ala Ala Gly Glu Ser Ala Phe
 80                  85                  90                  95

aga gtc aca ttt gat tgg gat gat gag gaa ttt gga gtt cca gga gca   875
Arg Val Thr Phe Asp Trp Asp Asp Glu Glu Phe Gly Val Pro Gly Ala
                100                 105                 110

ttc att atc aag aac ttg cat ttt agt gag ttc ttc ctc aag tca ctc   923
Phe Ile Ile Lys Asn Leu His Phe Ser Glu Phe Phe Leu Lys Ser Leu
            115                 120                 125

acc ctt gaa gat gtt cct aat cat ggc aaa gtt cat ttt gtc tgt aat   971
Thr Leu Glu Asp Val Pro Asn His Gly Lys Val His Phe Val Cys Asn
        130                 135                 140

tct tgg gtt tat cct gct aat aaa tat aag tca gat cgc atc ttc ttc  1019
Ser Trp Val Tyr Pro Ala Asn Lys Tyr Lys Ser Asp Arg Ile Phe Phe
    145                 150                 155

gcg aat cag gct tat cta cca agt gaa aca cca gac aca ttg cga aaa  1067
Ala Asn Gln Ala Tyr Leu Pro Ser Glu Thr Pro Asp Thr Leu Arg Lys
160                 165                 170                 175

tac aga gaa aat gaa tta gta acc tta aga gga gat gga act gga aag  1115
Tyr Arg Glu Asn Glu Leu Val Thr Leu Arg Gly Asp Gly Thr Gly Lys
                180                 185                 190

ctt gag gaa tgg gat aga gtt tat gac tat gct tac tac aat gac ttg  1163
Leu Glu Glu Trp Asp Arg Val Tyr Asp Tyr Ala Tyr Tyr Asn Asp Leu
            195                 200                 205

ggt gat cca gac aaa ggc caa gat ttg tca agg cct gtc tta gga gga  1211
Gly Asp Pro Asp Lys Gly Gln Asp Leu Ser Arg Pro Val Leu Gly Gly
        210                 215                 220

tct tct gag tac ccg tat cct cgt aga ggc agg aca ggc cgc aaa cca  1259
Ser Ser Glu Tyr Pro Tyr Pro Arg Arg Gly Arg Thr Gly Arg Lys Pro
    225                 230                 235

acc aaa aca gat cct aat tcc gag agc agg att cca ttg ctt atg agc  1307
Thr Lys Thr Asp Pro Asn Ser Glu Ser Arg Ile Pro Leu Leu Met Ser
240                 245                 250                 255

tta gac ata tat gtg cca agg gac gag cga ttt ggt cat ata aag ttg  1355
Leu Asp Ile Tyr Val Pro Arg Asp Glu Arg Phe Gly His Ile Lys Leu
                260                 265                 270

tca gac ttc ttg aca ttt gct ttg aaa tcc att gtg cag ttg ctt ctc  1403
Ser Asp Phe Leu Thr Phe Ala Leu Lys Ser Ile Val Gln Leu Leu Leu
            275                 280                 285

cct gag ttt aag gct ttg ttc gat agc acg cat aat gag ttt gat agt  1451
Pro Glu Phe Lys Ala Leu Phe Asp Ser Thr His Asn Glu Phe Asp Ser
        290                 295                 300

ttt gag gat gta ctt aaa ctg tat gaa gga gga atc aag ttg cct caa  1499
Phe Glu Asp Val Leu Lys Leu Tyr Glu Gly Gly Ile Lys Leu Pro Gln
    305                 310                 315

ggc cct ttg ttg aaa gcc att act gat agc att cct tta gag ata cta  1547
Gly Pro Leu Leu Lys Ala Ile Thr Asp Ser Ile Pro Leu Glu Ile Leu
```

```
320                 325                 330                 335

aaa gaa ctc ctt cga agt gat ggt gaa ggc cta ttt aag tac cca act   1595
Lys Glu Leu Leu Arg Ser Asp Gly Glu Gly Leu Phe Lys Tyr Pro Thr
                340                 345                 350

cct cag gtt att caa gag gat aaa act gca tgg agg acg gat gaa gaa   1643
Pro Gln Val Ile Gln Glu Asp Lys Thr Ala Trp Arg Thr Asp Glu Glu
            355                 360                 365

ttt ggg aga gaa atg ttg gcg gga gtc aat cct gtc ata atc agt aga   1691
Phe Gly Arg Glu Met Leu Ala Gly Val Asn Pro Val Ile Ile Ser Arg
        370                 375                 380

ctc caa gaa ttc cct ccg aaa agc aag ttg gat cct aaa ata tat ggc   1739
Leu Gln Glu Phe Pro Pro Lys Ser Lys Leu Asp Pro Lys Ile Tyr Gly
    385                 390                 395

aac caa aac agt aca att acc aga gag cag ata gag gat aag ttg gat   1787
Asn Gln Asn Ser Thr Ile Thr Arg Glu Gln Ile Glu Asp Lys Leu Asp
400                 405                 410                 415

gga cta aca att gat gag gca atc aag act aac aga cta ttc ata ttg   1835
Gly Leu Thr Ile Asp Glu Ala Ile Lys Thr Asn Arg Leu Phe Ile Leu
                420                 425                 430

aac cat cat gat atc ctt atg cca tac ttg agg aga att aac acg tcg   1883
Asn His His Asp Ile Leu Met Pro Tyr Leu Arg Arg Ile Asn Thr Ser
            435                 440                 445

aca gac aca aaa acc tat gcc tca aga act ctg ctc ttc ttg caa gat   1931
Thr Asp Thr Lys Thr Tyr Ala Ser Arg Thr Leu Leu Phe Leu Gln Asp
        450                 455                 460

aat gga act ttg aag cca tca gca att gaa cta agc ttg cca cat cca   1979
Asn Gly Thr Leu Lys Pro Ser Ala Ile Glu Leu Ser Leu Pro His Pro
    465                 470                 475

gac gga gat caa ttt ggc gct gtt agc aaa gta tat aca cca gct gat   2027
Asp Gly Asp Gln Phe Gly Ala Val Ser Lys Val Tyr Thr Pro Ala Asp
480                 485                 490                 495

caa ggt gtt gaa ggt tct atc tgg cag ttg gcc aaa gcc tat gca gca   2075
Gln Gly Val Glu Gly Ser Ile Trp Gln Leu Ala Lys Ala Tyr Ala Ala
                500                 505                 510

gtg aat gat tcg ggc gtt cat caa ctc atc agt cac tgg ttg aat aca   2123
Val Asn Asp Ser Gly Val His Gln Leu Ile Ser His Trp Leu Asn Thr
            515                 520                 525

cat gca gcg ata gag cca ttc gtg atc gca aca aat agg caa cta agc   2171
His Ala Ala Ile Glu Pro Phe Val Ile Ala Thr Asn Arg Gln Leu Ser
        530                 535                 540

gcg ctt cac cct att tat aag ctt ctc cac cct cat ttc cgt gag acg   2219
Ala Leu His Pro Ile Tyr Lys Leu Leu His Pro His Phe Arg Glu Thr
    545                 550                 555

atg aac ata aat gct tta gca aga cag atc ttg atc aac ggt ggt gga   2267
Met Asn Ile Asn Ala Leu Ala Arg Gln Ile Leu Ile Asn Gly Gly Gly
560                 565                 570                 575

ctt ctt gag ttg aca gtt ttt ccg gcc aaa tat tcc atg gaa atg tca   2315
```

```
Leu Leu Glu Leu Thr Val Phe Pro Ala Lys Tyr Ser Met Glu Met Ser
                580                 585                 590

gca gta gtt tac aaa gac tgg gtt ttc cct gaa caa gca ctt cct act   2363
Ala Val Val Tyr Lys Asp Trp Val Phe Pro Glu Gln Ala Leu Pro Thr
            595                 600                 605

gat ctc atc aaa aga gga gta gct gtt gag gac tcg agc tcc cca ctt   2411
Asp Leu Ile Lys Arg Gly Val Ala Val Glu Asp Ser Ser Ser Pro Leu
        610                 615                 620

ggc att cga tta ctg att cag gac tat cca tat gct gtt gat ggg ttg   2459
Gly Ile Arg Leu Leu Ile Gln Asp Tyr Pro Tyr Ala Val Asp Gly Leu
    625                 630                 635

aaa att tgg tca gca att aaa agt tgg gta act gaa tac tgc aac tac   2507
Lys Ile Trp Ser Ala Ile Lys Ser Trp Val Thr Glu Tyr Cys Asn Tyr
640                 645                 650                 655

tat tac aaa tca gat gat gcg gtt caa aaa gac act gaa ctc caa gcc   2555
Tyr Tyr Lys Ser Asp Asp Ala Val Gln Lys Asp Thr Glu Leu Gln Ala
                660                 665                 670

tgg tgg aag gaa ctc cgc gaa gag gga cac ggt gac aag aaa gat gag   2603
Trp Trp Lys Glu Leu Arg Glu Glu Gly His Gly Asp Lys Lys Asp Glu
            675                 680                 685

cct tgg tgg cct aaa atg cag aca gtg caa gaa ttg ata gac tct tgc   2651
Pro Trp Trp Pro Lys Met Gln Thr Val Gln Glu Leu Ile Asp Ser Cys
        690                 695                 700

acc atc aca ata tgg ata gct tca gca ctt cat gca gca gtc aat ttc   2699
Thr Ile Thr Ile Trp Ile Ala Ser Ala Leu His Ala Ala Val Asn Phe
    705                 710                 715

ggg caa tac cct tat gct ggt tat ctc cct aat cgc cct aca tta agc   2747
Gly Gln Tyr Pro Tyr Ala Gly Tyr Leu Pro Asn Arg Pro Thr Leu Ser
720                 725                 730                 735

cga aat ttc atg cca gag cca gga agt cct gag tat gaa gag ctc aag   2795
Arg Asn Phe Met Pro Glu Pro Gly Ser Pro Glu Tyr Glu Glu Leu Lys
                740                 745                 750

aca aat ccg gat aag gta ttc ctc aaa aca atc act cct cag ctg cag   2843
Thr Asn Pro Asp Lys Val Phe Leu Lys Thr Ile Thr Pro Gln Leu Gln
            755                 760                 765

aca ctg ctt ggc att tcc ctc ata gag atc ttg tca agg cat tct tcg   2891
Thr Leu Leu Gly Ile Ser Leu Ile Glu Ile Leu Ser Arg His Ser Ser
        770                 775                 780

gat aca ctt tac ctc ggg caa agg gaa tca cct gaa tgg aca aag gat   2939
Asp Thr Leu Tyr Leu Gly Gln Arg Glu Ser Pro Glu Trp Thr Lys Asp
    785                 790                 795

caa gaa cca ctt tca gct ttt gcg agg ttt gga aag aag ctg agt gat   2987
Gln Glu Pro Leu Ser Ala Phe Ala Arg Phe Gly Lys Lys Leu Ser Asp
800                 805                 810                 815

atc gag gat cag att atg cag atg aat gtc gat gag aaa tgg aag aac   3035
Ile Glu Asp Gln Ile Met Gln Met Asn Val Asp Glu Lys Trp Lys Asn
                820                 825                 830
```

agg tcg ggt cct gtt aaa gtt cca tac acc ttg ctc ttc ccc aca agt 3083
Arg Ser Gly Pro Val Lys Val Pro Tyr Thr Leu Leu Phe Pro Thr Ser
835 840 845

gaa gga gga ctt act ggc aaa gga att cct aac agt gtg tca ata tag 3131
Glu Gly Gly Leu Thr Gly Lys Gly Ile Pro Asn Ser Val Ser Ile
850 855 860

aactttcccg atctagtaac atagatgaca ccgcgcgcga taatttatcc tagtttgcgc 3191

gctatatttt gttttctatc gcgtattaaa tgtataattg cgggactcta atcataaaaa 3251

cccatctcat aaataacgtc atgcattaca tgttaattat tacatgctta acgtaattca 3311

acagaaatta tatgataatc atcgcaagac cggcaacagg attcaatctt aagaaacttt 3371

attgccaaat gtttgaacg 3390

<210> 3
<211> 30
<212> DNA
<213> Artificial sequence

<220>
<223> Artificial sequence description: Sense primer

<400> 3
gttatcaaac agtttaaaat gtttctggag 30

<210> 4
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> Artificial sequence description: Reverse primer

<400> 4
tgatttaaag ttctatattg ac 22

<210> 5
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Artificial sequence description: Primer F

<400> 5
ggccatggag tcaaagattc 20

<210> 6
<211> 19
<212> DNA
<213> Artificial sequence

<220>

<223> Artificial sequence description: Primer R

<400> 6
ggtctggcat gaaatttcg 19